JOURNAL OF BACTERIOLOGY, Mar. 2001, p. 1780–1783 Vol. 183, No. 5
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.5.1780–1783.2001

NOTES

Role for *draTG* and *rnf* Genes in Reduction of 2,4-Dinitrophenol by *Rhodobacter capsulatus*

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Received 28 July 2000/Accepted 2 December 2000

The phototrophic bacterium *Rhodobacter capsulatus* is able to reduce 2,4-dinitrophenol (DNP) to 2-amino-4-nitrophenol enzymatically and thus can grow in the presence of this uncoupler. DNP reduction was switched off by glutamine or ammonium, but this short-term regulation did not take place in a *draTG* deletion mutant. Nevertheless, the target of DraTG does not seem to be the nitrophenol reductase itself since the ammonium shock did not inactivate the enzyme. In addition to this short-term regulation, ammonium or glutamine repressed the DNP reduction system. Mutants of *R. capsulatus* affected in *ntrC* or *rpoN* exhibited a 10-fold decrease in nitroreductase activity in vitro but almost no DNP activity in vivo. In addition, mutants affected in *rnfA* or *rnfC*, which are also under NtrC control and encode components involved in electron transfer to nitrogenase, were unable to metabolize DNP. These results indicate that NtrC regulates dinitrophenol reduction in *R. capsulatus*, either directly or indirectly, by controlling expression of the Rnf proteins. Therefore, the Rnf complex seems to supply electrons for both nitrogen fixation and DNP reduction.

The industrial production and abusive use of dyes, explosives, herbicides, pesticides, and drugs result in the release of nitroaromatic compounds into the environment (26). These xenobiotic compounds are resistant to oxygenolytic reactions since the nitroaromatic ring is rendered impervious to electrophilic attack, especially in the case of polynitroaromatics. Therefore, microorganisms have developed reductive pathways that facilitate the metabolism of these recalcitrant compounds. The process may began with reduction of the aromatic ring (5, 18) or with reduction of the nitro group to the corresponding amino or hydroxylamino derivatives that can be assimilated upon release of ammonium and hydroxyaromatic adducts (11, 24).

Under light and anaerobiosis, *Rhodobacter capsulatus* cometabolizes the uncoupler 2,4-dinitrophenol (DNP) by reducing it to 2-amino-4-nitrophenol, which is almost stoichiometrically accumulated in the medium (2). The reaction is catalyzed by a cytosolic and homodimeric Flavin mononucleotide-linked, 54-kDa nitrophenol reductase (NPR) (3). Once DNP is consumed, the cells began to grow by fixing the dinitrogen dissolved in the medium.

The reduction of DNP in *R. capsulatus* is repressed by ammonium since the process does not takes place in ammonium-grown cells in the presence of chloramphenicol (2). To assess if the reduction of DNP is activated in N_2-fixing cultures, cells cultured as previously described (2) with glutamate (nitrogenase-derepressing conditions), ammonium (negative control), and glutamate plus DNP (positive control) as nitrogen sources were transferred to media with DNP. DNP consumption and NPR activity were determined as published elsewhere (3). As expected, the maximal rate of DNP photoreduction was observed in cells previously cultured with DNP (Fig. 1), showing a NPR activity of 3.2 ± 0.5 mU mg^{-1}. The consumption of DNP by these cells was independent of the presence of chloramphenicol (not shown). If the cells were precultured with glutamate, the rate of DNP reduction was lower (Fig. 1). The NPR activity of extracts from these cells was 0.3 ± 0.02 mU mg^{-1}. In this case, the addition of chloramphenicol inhibited DNP uptake and reduction, which completely ceased after 2 to 3 h incubation (not shown). In contrast, the ammonium-grown cells started to photoreduce DNP after a period of 3 h (Fig. 1), and NPR activity was very low (0.03 ± 0.01 mU mg^{-1}). In this case, DNP reduction does not take place in the presence of chloramphenicol (2). These results suggest that the pathway for DNP metabolism (transport and reduction) is completely repressed in the presence of ammonium, whereas under nitrogenase-derepressing conditions (with glutamate or dinitrogen) the pathway is fully activated only if the substrate DNP is present. The activating effect of DNP was dependent on de novo protein synthesis and can be explained taking into account the lability of the enzyme in the absence of DNP (6). This conclusion was corroborated by staining the diaphorase activity of the NPR protein in nondenaturing pore-gradient polyacrylamide gel electrophoresis and by two-dimensional gel electrophoresis (not shown).

The regulation pattern of DNP reduction by ammonium resembles that described for nitrogen fixation in *R. capsulatus*. The expression of Nif (nitrogen fixation) proteins in *R. capsulatus* is enhanced by a high intracellular [2-oxoglutarate]/[glu-

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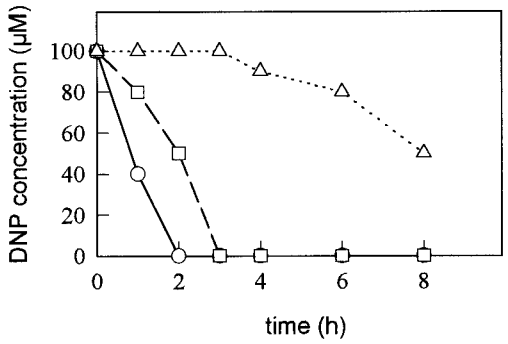

FIG. 1. Time course of DNP photoreduction by *R. capsulatus* cultured with different nitrogen sources. Cells grown with ammonium-acetate (△), glutamate-acetate (□) and glutamate-DNP-acetate (○) were washed and incubated with DNP-acetate. DNP concentration was determined in aliquots taken from the cultures at the times indicated, 2-amino-4-nitrophenol produced was almost stoichiometric with the DNP consumed (not shown). Data are from a single experiment; two independent experiments yielded essentially the same results.

tamine] ratio, which activates a cascade mechanism that includes the NtrC-NtrB (NifR1-NifR2) two-component regulatory system, which activates its target genes (e.g., *nifA*) in concert with RNA polymerase containing the housekeeping σ^{70} factor (9). Expression of *nif* genes is then activated by NifA, which interacts with RNA polymerase harboring σ^{54} (RpoN) (15, 16, 17, 19). The Ntr system detects the oscillations of the glutamine concentration inside the cells, so that the irreversible inhibition of glutamine synthetase by L-methionine-SR-sulfoximine (MSX) prevents the inorganic nitrogen metabolism from being regulated by ammonium (1, 10).

To study the regulatory links between nitrogen fixation and DNP photoreduction, we examined some mutants affected in nitrogen fixation (19).

The *R. capsulatus* mutants used were defective in the nitrogenase structural genes (*nifHDK*), in regulatory components (*ntrC* and *poN*), and in genes encoding proteins involved in electron transport to nitrogenase (*nifF*, *rnfF*, *rnfA*, *rnfC*, and *orf14*) (13, 25). Deletion of the nitrogenase structural genes did not affect DNP photoreduction (Table 1). NifF is a flavodoxin required for electron transfer to nitrogenase under iron deprivation (27). The molecular properties of this protein resemble those of NPR (3), but *nifF* mutants showed an NPR^+ phenotype (Table 1). In addition, NPR did not react against NifF antibodies (data not shown), indicating that NifF and NPR are two different proteins. The *rnf* cluster consists of two operons, both essential for nitrogen fixation in the light (25) or dark (23). One *rnf* operon (*orf14*, *fdxC*, *fdxN*, *rnfF*, and *orf10*) does not seem to be involved in supplying electrons to NPR since mutants with insertion mutations in *orf14* (with the interposon integrated in both directions) and in *rnfF* reduced DNP to the same extent as the wild type (Table 1). By contrast, strains mutated in two genes of the other *rnf* operon (*rnfA* and *rnfC*) were almost unable to reduce DNP in vivo and showed a constitutive NPR activity around 10-fold lower than that of the wild-type strain (Table 1). The *ntr* regulatory *ntrC* and *rpoN* mutants showed a phenotype similar to that of the *rnfA* and *rnfC* mutants (Table 1). All mutants affected in the reduction of DNP in vivo became more sensitive to DNP, showing a lag phase of 85 h in the presence of the uncoupler (not shown). The residual DNP reduction observed in vivo in the *rnf* and *ntr* mutants (20% of the wild-type level) could be due to unspecific nitroreductases similar to those found in other bacteria (7).

TABLE 1. Effect of ammonium on metabolism of DNP and NPR activities in *R. capsulatus* wild-type and mutant strains.

R. capsulatus Strain	Genotype	NPR activity (mU mg^{-1})		% DNP consumed[a]
		$-NH_4^+$	$+NH_4^+$	
E1F1	Wild type	2.8 ± 0.3	0.04 ± 0.01	100 ± 10
B10S	Wild type	2.9 ± 0.3	0.03 ± 0.01	100 ± 14
B10S mutant[b]	*nifHDK*	3.0 ± 0.2	0.04 ± 0.02	100 ± 15
	ntrC	0.4 ± 0.2	0.3 ± 0.2	20 ± 14
	rpoN	0.20 ± 0.01	0.20 ± 0.02	18 ± 10
	nifF	2.5 ± 0.2	0.02 ± 0.01	100 ± 12
	rnfF	2.4 ± 0.1	0.03 ± 0.02	100 ± 11
	rnfA	0.15 ± 0.01	0.17 ± 0.02	21 ± 15
	rnfC	0.20 ± 0.01	0.20 ± 0.01	23 ± 13
	orf14	2.5 ± 0.1	0.03 ± 0.02	100 ± 15

[a] Cells cultured in the presence of DNP were harvested by centrifugation in the mid-log phase and resuspended in medium with DNP; 100% corresponded to 0.2 mM DNP consumed in 6 h by the wild type. The data represent averages of four independent experiments.

[b] Previously described (24).

Since the expression of *rnf* genes is dependent on the Ntr system, DNP photoreduction is regulated either directly (by controlling the expression of NPR) or indirectly (through expression of the *rnf* genes). We propose an indirect control because relatively high in vitro NPR activities could be detected in crude extracts of *ntr* and *rnf* mutant strains (Table 1). Nevertheless, the NPR activity in the *ntr* and *rnf* mutants cultured in the presence of DNP was significantly lower than that observed in the wild-type strain. This fact could be explained taking into account that the NPR of *R. capsulatus* is very unstable in vitro when it became exposed to blue light in the absence of NADPH (6). Therefore, mutants affected in electron supply may be unable to maintain the enzyme in an appropriate reduced state to avoid photoinactivation.

In addition to the long-term effect of ammonium on the reduction of DNP described above, ammonium addition causes a rapid and reversible inhibition of DNP photoreduction in *R. capsulatus* (2) (Fig. 2A). A similar inhibition pattern was observed by glutamine addition, but other amino acids such as glutamate did not inhibit DNP metabolism (not shown). This effect is achieved probably not by ammonium *per se* since (i) in phototrophic bacteria, ammonium shock increases the glutamine concentration inside the cells, which activates regulatory processes that inhibit nitrogen fixation (14) and nitrate assimilation (10), (ii) MSX relieved the ammonium suppression of DNP metabolism (4), and (iii) DNP reduction was rapidly and reversibly inhibited by addition of either ammonium or glutamine. This short-term inhibition of DNP metabolism resembles the effect described for nitrate transport or nitrogen fixation, which are switched off by ammonium addition in *Rhodospirillaceae* (8, 14, 27, 28). Nitrogen fixation in these bacteria is short-term regulated by ammonium by reversible ADP-ribosylation of the nitrogenase, catalyzed by the DraT and DraG proteins (12, 22). To test if the short-term effect of ammonium on DNP metabolism was mediated by the DraTG system that regulates nitrogenase activity in *R. capsulatus*, we analyzed a mutant defective in *draTG* (20) and found

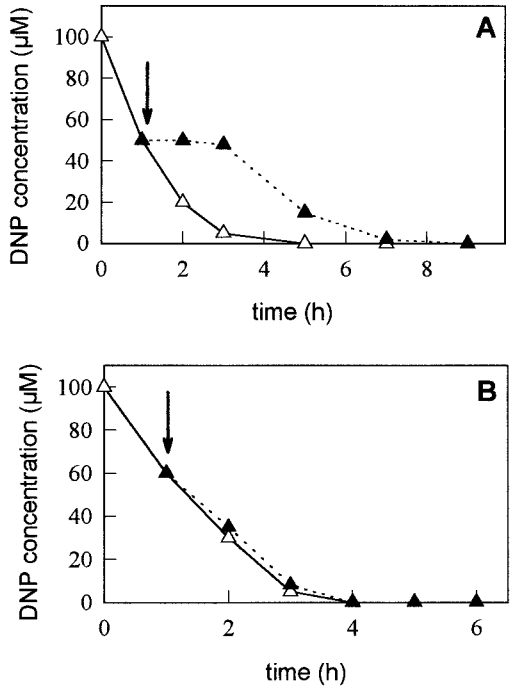

FIG. 2. Effect of ammonium on DNP metabolism in *R. capsulatus* wild-type (A) and *draTG* double-deletion mutant (B) strains. Cells grown with 0.1 mM DNP were harvested by centrifugation and resuspended in fresh medium with 10 mM acetate and 0.1 mM DNP. The flasks were incubated under light at 30°C, and DNP concentration was determined. At the time indicated by arrows, half of each culture was made 2 mM in NH_4Cl (filled symbols), whereas the other half remained under the same growth conditions (open symbols). Data are representatives of three different experiments.

that ammonium did not repress DNP reduction in this mutant (Fig. 2B). Therefore, both nitrogen fixation and DNP reduction are regulated on the posttranslational level via DraTG (Fig. 2). In contrast to nitrogenase, which is modified at residue Arg^{102} of the NifH protein and thus is inactivated, the activity of NPR itself is not suppressed. It is interesting that an *R. capsulatus* strain carrying an NifH-Arg^{102} substitution mutant still showed ammonium switch-off (21), indicating a second, not yet identified target for DraT-dependent regulation. Since nitrogen fixation and DNP reduction share the *rnf*-encoded electron transfer system, one of the Rnf proteins might be this target.

In conclusion, both nitrogen fixation and DNP reduction in *R. capsulatus* need the low level reduction power provided by the Rnf system. The Ntr control of *rnf* expression could explain the long-term (transcriptional) regulation of DNP reduction by ammonium. In addition, we found that the short-term regulatory effect of ammonium on DNP reduction depended on the DraTG system. Since NPR itself was shown not to be the target of ADP-ribosylation, we speculated that either the electron transfer system encoded by *rnf* or the DNP uptake system may be controlled by DraTG.

We acknowledge financial support from DGICYT (grant PB 95 0554 CO2 02) and PAI (grant CVI 0117) (Spain) and the Alexander von Humboldt Foundation (Germany). R.B. acknowledges financial support from the MEC (Contratos de Incorporación de Doctores y Tecnólogos).

We thank Alexander Yakunin and Patrick Hallenbeck for providing the antibodies against NifF and C. Moreno-Vivián for critically reading the manuscript.

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